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CELL-SIZED, SUPPORTED ARTIFICIAL MEMBRANES (PSEUDOCYTES): RESPONSE OF PRECURSOR CYTOTOXIC T LYMPHOCYTES TO CLASS I MHC PROTEINS[1]

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Novel cell-sized, supported artificial membranes bearing class I antigens have been prepared by a simple dialysis procedure and then used to study the requirements for antigen recognition by precursor cytotoxic T lymphocytes (CTL). The membranes were made by mixing lipid, H-2 antigen, and C_{18} alkylated 5 μm silica beads in deoxycholate, and dialyzing to remove the detergent. The H-2 antigen-bearing, cell-sized beads, termed pseudocytes (artificial cells), were able to simulate generation of secondary CTL responses with the same specificity as alloantigen-bearing spleen cells. Comparative analyses demonstrated that the size of an antigen-bearing structure, and thus its potential for multivalent interaction, was a critical determinant of effectiveness of antigen recognition, and showed that H-2 antigen was recognized as effectively on cell-sized beads as on allogeneic spleen cells.

Generation of a response to antigen on the cell-sized beads was completely dependent on addition of lymphokines to the cultures. Thus, unlike liposomes, H-2 antigen on beads was not available to accessory cells for stimulation of Ia-dependent production of lymphokines by T helper cells. These results, as well as direct observations by microscopy, strongly indicate that antigen is recognized on the surface of the beads. Despite effective stimulation of secondary CTL responses, antigen on beads was completely inactive in stimulating a primary CTL response by naive spleen cells. The results of mixing experiments by using beads and alloantigen-bearing cells or plasma membrane vesicles indicate that the lack of a primary response may result from a requirement for a soluble factor(s) that is not needed for generation of secondary responses.

The unique advantages of cell-size supported membranes for studying antigen recognition by T cells are discussed. The beads can be handled and used like antigen-bearing cells in functional assays, while possessing well-defined, readily varied, and easily quantitated composition.

Generation of virtually all immune system responses requires T cell recognition of foreign antigens and major histocompatibility complex (MHC) proteins on the surfaces of other cells (1–3). Although the functional consequences of T cell antigen recognition are known in considerable detail, and many lymphocyte surface molecules involved in antigen-specific responses have been described at both the protein and nucleic acid level, the molecular interactions occurring at the cell surface that lead to response are poorly understood. These T cell recognition events occur at the interface between cells and involve dynamic and highly multivalent interactions between membrane-bound receptors and ligands, and precise definition, measurement, modification, or control of molecules on the surfaces of live cells is difficult at best.

Replacing antigen-bearing cells with well-defined artificial membranes provides one approach to better determine the parameters that are important during recognition and triggering of responses. The use of antigen-bearing liposomes, for example, has provided detailed information about antigen structure (4) and soluble mediator (5) requirements for activation of precursor cytotoxic T lymphocytes (CTL), and demonstrated that multivalent interaction with antigen is critical to triggering of these precursor cells (6). However, the results also indicate that the small size of liposomes (<0.1 μm) places severe limitations on their recognition (6), which is consistent with the observation that CTL interact with target cells over relatively large areas of the cell surface (7). Furthermore, the state of liposomes at the time of their recognition is difficult to determine. Antigen-bearing liposomes are readily taken up by accessory cells in culture, as demonstrated by the fact that they can stimulate Ia-dependent T helper cell production of lymphokines (8). Although there is considerable evidence to indicate that precursor CTL triggering can result from direct recognition of the antigen on the liposome membrane (6), rather than by cellular presentation, this has not been directly demonstrated. Some of these difficulties can be overcome by using antigen-bearing membranes supported on glass slides or 100 μm beads, which have recently been shown to allow triggering of precursor CTL (9) and helper T cell hybridomas (10, 11), and to permit the use of biophysical methods to study T cell receptor interaction with antigen (12). At the same time, there are significant limitations on the ability to characterize such membranes and to use them in functional studies.

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This report describes a new type of artificial membrane system, antigen-bearing lipid membranes supported on cell-sized, silica-based beads, and demonstrates their unique advantages for the study of T cell recognition. Antigen is incorporated onto the bead-supported membranes in a controlled and measurable manner by dialysis, and remains both serologically and immunologically active. The cell-sized particles allow for extensive surface interactions with cells, while being readily amenable to characterization by fluorescence-activated cell sorter analysis (manuscript in preparation) and by incorporation of radiolabeled lipid and protein. The composition of the bead membranes can be easily varied, small amounts of antigen are required to prepare large number of beads (6 μg per 10^7 beads), and the particles provide the flexibility of handling needed for detailed examination of functional recognition. Finally, they are easily seen under the microscope and are readily distinguished from cells, so that direct visualization and assessment of lymphocyte-antigen interaction is simply accomplished. Because the bead-supported membranes are cell-size, can be handled like antigen-bearing cells to reconstitute functional responses normally dependent on cell-cell recognition, and are as specific and potent as intact stimulator cells in triggering antigen-dependent primed precursor CTL responses, we propose that they be called "pseudocytes" (artificial cells).

MATERIALS AND METHODS

Mice and tumor cells. (BALB/c × DBA/2)F_1 (CD2F_1) (H-2^d), (AKR × DBA/2)F_1 (AKD2F_1) (H-2^{kxd}), C57BL/6 (H-2^b), AKR/J (H-2^k), and A/J (H-2^a) mice were purchased from The Jackson Laboratory, Bar Harbor, ME. RDM-4 (H-2^k), an AKR lymphoma, P815 (H-2^d), a mastocytoma of DBA/2 origin, and EL-4 (H-2^b), a thymoma of C57BL/6 origin, were maintained in tissue culture and by passage as ascites.

Purification of class I antigens. H-2K^k was purified from RDM-4 ascites cell detergent lysates by affinity chromatography on an 11-4.1 monoclonal antibody (mAb)[4] column as described (13), with a yield of approximately 0.9 mg H-2K^k/10^{10} cells. H-2K^d/D^d and H-2K^b/D^b antigens were purified by affinity chromatography from P815 or EL-4 ascites cell detergent lysates respectively on an M1/42 mAb column as described (14) with a yield of approximately 0.25 mg H-2 antigen/10^{10} cells. All H-2 preparations were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 5 to 15% acrylamide slab gels by using the buffer system of Laemmli (15) with a 3% stacking gel. Protein was visualized by staining with Coomassie Brilliant Blue. ^{125}I-H-2K^k was prepared by reacting 10 μg purified antigen with 0.5 mCi Na^{125}I (New England Nuclear, Boston, MA) and two Iodobeads (Pierce Chemical Corp., Rockford, IL) in 0.15 ml 0.5% deoxycholate for 30 min at 4°C, and was isolated by Sephadex G-25 chromatography and dialysis, yielding a product with a specific activity of about 300 cpm/ng protein.

Preparation and handling of pseudocytes. Spherisorb 5 μm ODS1 beads were purchased from Phase Sep, Norwalk, CT. Dry beads were suspended by vortex mixing in 0.5% deoxycholate (DOC) in 10 mM Tris buffered-saline (TBS), 0.14 M NaCl, pH 8.0 (DOC/TBS), were washed twice in this buffer by centrifugation (2 min, 1500 rpm), and were counted by using a hemocytometer. Lipids were either derived by chloroform/methanol extraction of P815 cells (cellular lipid) and then quantitation based on phosphate as described (16), or were purchased from Sigma Chemical Co. (St. Louis, MO) and then used without additional purification. After evaporation of organic solvents and solubilization in DOC/TBS, synthetic lipids were mixed in a ratio of 98:2, dimyristoylphosphatidylcholine (DMPC):cholesterol (chromatography quality). ^{3}H-dipalmitoylphosphatidylcholine (60 Ci/mmol) was purchased from New England Nuclear.

Components were incorporated onto the beads by mixing H-2 antigen, lipid, and the 5 μm beads in DOC/TBS and dialyzing at 4°C for 36 to 48 hr to remove the detergent. Unless otherwise noted, the ratios used were 6 μg H-2K^k or 12 μg H-2^d or H-2^b per 10^7 beads and 5 nmol lipid per 10^7 beads, with 2 to 20 × 10^6 beads in 0.75 ml buffer. Dialysis was against 0.6 L TBS in a sterile tissue culture flask containing SM-2 biobeads (BioRad, Richmond, CA), prepared according to the manufacturer's directions and used at 1 g per ml of sample being dialyzed, as a detergent absorbant. The flask was placed on a rotating platform (Roto Torque Rotator; Cole Palmer, Chicago, IL) to keep the 5 μm beads in suspension during the entire period of dialysis. After 24 hr of dialysis, 5 mM $CaCl_2$ was added to the dialysis buffer. Dialysis tubing (Spectra/Por 2 membranes, 12–14,000 MWCO; Fischer Scientific, Phoenix, AZ) was treated for 1 min in boiling water and closed with clips (Spectra/Por closures; Fischer) after sample addition to completely exclude air bubbles from the bag. Complete exclusion of air is necessary or beads and lipid accumulate at the air-fluid interface. After dialysis, the dialysis bag was cut open, and the beads were removed and washed three times in sterile medium and then stored at 4°C until added to culture.

The beads are well behaved in serum-containing medium and can be vortexed, transferred by plastic pipet, and collected by brief (1 to 2 min) low speed centrifugation without losses, and can be counted by using a hemocytometer. When handled in protein-free buffer such as in the papain cleavage experiments detailed below, it is important to prevent exposure of the particles to an air-fluid interface. Thus, vortex mixing and complete removal of fluid after centrifugation are avoided.

Papain cleavage. Exposure of incorporated antigen at the bead surface was assessed by susceptibility to papain cleavage. For cleavage experiments, the beads were harvested without added serum, were washed three times in TBS, and were divided into aliquots of 7 × 10^5 beads (10,000 cpm ^{125}I-H-2K^k, 0.42 μg H-2K^k) for treatment with 0.33 U/sample soluble papain (Type IV from papaya latex; Sigma), 1 U/sample papain immobilized on carboxymethylcellulose beads (Sigma), or no enzyme. The immobilized enzyme was washed three times in TBS before preactivation. Enzyme was preactivated for 30 min at 37°C with 50 mM cysteine, and 5 mM cysteine was present during the cleavage. After cleavage, beads were pelleted by centrifugation and the ^{125}I-H-2 radioactivity in the supernatant fluid and pellets was determined. Cleavage for 90 min was found to release 80% as much radioactivity as was solubilized by overnight treatment.

Preparation of liposomes and liposome-coated beads. Purified H-2K^k was incorporated into unilamellar liposomes by mixing with extracted cellular lipid (1 μg H-2K^k/20 nmol lipid) in DOC/TBS, followed by dialysis to remove the detergent, as described (16), except that liposomes were not harvested by centrifugation. Twenty-four hours of dialysis against TBS was followed by 16 hr against 5 mM $CaCl_2$, 10 mM HEPES, 0.14 M NaCl, pH 7.9, and this stock solution of liposomes was used directly. Liposome-coated beads were prepared by mixing previously formed liposomes containing 7 μg H-2K^k with 10^7 5 μm beads (washed in absolute ethanol and dried before use) and incubating at 37°C for 60 min with intermittent vortexing, followed by three washes in serum-containing medium.

In vitro CTL generation and ^{51}Cr-release assay. Alloantigen-bearing beads, liposomes, or adherent cell-depleted allogeneic splenocytes were added to responder splenocytes from CD2F_1 mice primed 2 to 6 mo previously by i.p. injection of 2 × 10^7 RDM-4 (H-2^k) cells and cultured in 2 ml of medium with 7 × 10^6 cells/well (Linbro, New Haven, CT) at 37°C in 5% CO_2 for H-2^d anti-H-2^k responses. For H-2^k anti-H-2^d responses, responder splenocytes were from AKR/J mice primed 2 to 6 mo previously by i.p. injection of 2 × 10^7 P815 (H-2^d) cells. For H-2^b anti-H-2^k responses, responder splenocytes were from C57BL/6 mice primed 2 to 6 mo previously by i.p. injection of 2 × 10^7 RDM-4 cells. Depletion of adherent cells was performed by incubating for 90 min at 10^7/ml and 5 ml/60 mm tissue culture-treated dish, followed by overnight culture of the nonadherent cells and irradiation (3000 R) immediately before use as stimulators. An optimal concentration of an $(NH_4)_2SO_4$ fraction of supernatant fluid from rat spleen cells cultured with concanavalin A (Con A supernatant), prepared as described (5), was added at 22 hr of culture unless otherwise indicated. All experiments included control cultures lacking alloantigen. The culture medium used was RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 50 μM 2-mercaptoethanol, and 5 mg gentamycin/dl.

Nonimmune CD_2F_1 responder splenocytes were cultured in the same manner as described for immune populations. When C57BL/6 or A/J splenocytes were included as third party stimulators, they were irradiated (3000 R) and added at 10^6/culture. When RDM-4 plasma membrane vesicles (PMV) were included in cultures, they were prepared as described (17) and were added at 2.5 μg PMV protein/ml culture fluid.

After 5 days, each culture was assayed for cytotoxicity at several

[4] Abbreviations used in this paper: DMPC, dimyristoylphosphatidylcholine; DOC, deoxycholate; mAb, monoclonal antibody; PMV, plasma membrane vesicle; STEM, scanning transmission electron microscopy; TBS, Tris-buffered saline.

effector to target cell ratios by triplicate determination of the ^{51}Cr released in 4 hr from 10^4 labeled RDM-4 or P815 target cells incubated at 37°C in 5% CO_2. The percent specific ^{51}Cr release = 100 × (experimental − spontaneous ^{51}Cr release)/(total released by 0.5% DOC − spontaneous ^{51}Cr release). Spontaneous release was less than 15% for all experiments. Lytic units provide an estimate of the relative number of effector CTL in a population (18). One lytic unit is defined as the number of cultured cells required to lyse 50% of the target cells in the 4-hr ^{51}Cr-release assay, and are reported as lytic units per 10^6 responder cells.

Pseudocyte binding and light microscopy. Effector CTL cultures resulting from 5 day stimulation with 1.5×10^6 pseudocytes, as described above, were washed three times by centrifugation in culture medium, were resuspended, and were viewed microscopically. The cells were split into aliquots for incubation with either anti-H-$2K^k$ mAb (11-4.1) or an irrelevant anti-NP idiotype mAb (R3-I), generously donated by Dr. Carol Cowing, Medical Biology Institute, La Jolla, CA. Both antibodies are γ_{2a} subtype. Incubation for 30 min at room temperature of 5 μg mAb and 5×10^5 cells was followed by three washes and staining with 0.5 μg/sample of a fluoresceinated goat-anti-murine γ_{2a} antisera, generously donated by Dr. Donald Mosier, Medical Biology Institute. All antibodies were purified by ammonium sulfate fractionation and were clarified by centrifugation in an airfuge for 5 min at 100,000 × G and 4°C. After three additional washes, cells were viewed at a magnification of 400×, and light and fluorescent photography was done by using Kodak Tri-X film pushed to ASA 1200.

Scanning transmission electron microscopy. Untreated 5 μm beads (in DOC/TBS), or beads coated by dialysis with lipid or lipid and H-2 (in Dulbecco's phosphate-buffered saline, PBS), were allowed to settle by gravity onto glass coverslips coated with 1 mg/ml poly-L-lysine. Coverslips were then washed with Dulbecco's PBS and the samples were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 20 to 30 min. After a brief rinse, samples were postfixed in 1% OsO_4 for 20 min, were rinsed with buffer, were treated with a saturated solution of tannic acid for 5 min, were rinsed again, and were additionally fixed with 1% OsO_4. After a graded series of ethanol washes from 50 to 100% (5 min each), samples were placed in liquid freon 113 and were dried by substitution from freon to liquid CO_2 and then to dry CO_2 in a Polaron 3000 critical point dryer. Dried coverslips were coated by using a gold-palladium (80:20) source in an E5100 Polaron sputter coater. Samples were scanned by using a pointed filament in the secondary mode of an Hitachi H-600 scanning-transmission electron microscope (19).

RESULTS

Formation of cell-sized, supported artificial membranes (pseudocytes). As a solid support, we have used commercially available 5 μm silica-based spherical particles, covalently modified with C_{18} alkyl chains, which are sold as packing material for high pressure liquid chromatography reverse phase columns. Mixing of purified H-2 antigen, lipid, and beads in DOC, followed by dialysis to remove the detergent, results in association of all the lipid and protein with the beads, as assessed by incorporation of radiolabeled H-2 and lipid (Table I and Fig. 1). The resulting preparation consists of a fine suspension of individual beads that settles to the bottom of a tube on standing for 15 min. In 10 experiments, H-2 incorporation for a standard preparation (Table I, sample 1) varied by less than 5%.

TABLE I

H-2 antigen incorporation onto beads and release by papain treatment[a]

Sample	nmol Lipid[b]	% H-2 Bound[c]	% H-2 Released[c]	
			Soluble papain	Immobilized papain
1	5 cellular	92	42	36
2	50 cellular	87	40	42
3	5 synthetic	94	41	37
4	50 synthetic	97	48	45

[a] The indicated amount of lipid was mixed with 6 μg H-$2K^k$ and 10^7 5 μm beads in DOC/TBS and was dialyzed as described in *Materials and Methods*.

[b] Lipids used were: cellular, a chloroform:methanol extract of P815 tumor cells, or synthetic lipids (98:2, DMPC:cholesterol).

[c] Incorporation and release of H-2 antigen was monitored by including ^{125}I-H-$2K^k$ of known specific activity during coating of the beads. Papain cleavage was performed as described in *Materials and Methods*.

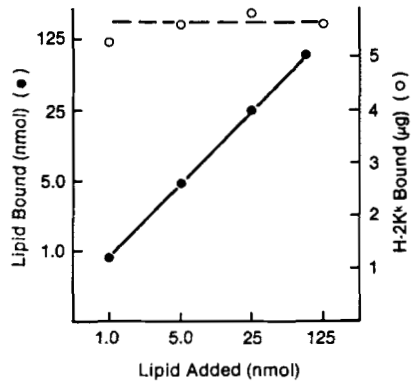

Figure 1. Incorporation of lipid and H-2 antigen onto beads by dialysis. H-$2K^k$ antigen (6 μg) and 10^7 beads were mixed with varying amounts of cell lipid in DOC/TBS, and were dialyzed as described in *Materials and Methods*. Trace amounts of ^3H-dipalmitoylphosphatidylcholine (PC) or ^{125}I-H-$2K^k$ were added to monitor incorporation onto the beads. After dialysis the beads were washed twice in serum-containing medium and radioactivity associated with the bead pellet was determined. In the absence of added beads, no radioactivity is recovered as a pellet. (○), ^{125}I-H-$2K^k$; (●), ^3H-PC.

When either synthetic lipids or extracted cellular lipids are used, approximately 40% of the H-2 antigen is exposed on the surface of the beads, as demonstrated by its cleavage by papain (Table I). Similar amounts of H-2 antigen are released by cleavage with either soluble papain or papain immobilized on a solid support. Thus, antigen is readily accessible at the bead surface, even to immobilized enzyme, and is therefore likely to be readily available for cellular recognition. Figure 1 shows that all added lipid coats the hydrophobic particles, and that changing the total amount of incorporated lipid does not effect the extent of H-2 antigen incorporation nor the fraction of H-2 antigen exposed on the surface and accessible to papain cleavage (Table I). If lipid is not present during preparation of the beads, they aggregate extensively and cannot be worked with after detergent removal.

The untreated 5 μm silica beads are uniformly coupled with C_{18} alkyl chains on all exposed surfaces and have pores that are 50 to 110 Å in diameter, seen as depressions on the surface of beads visualized by scanning transmission electron microscopy (STEM; Fig. 2*A* and *B*). The surfaces of the beads dialyzed with lipid and H-2 antigen appear slightly smoother (Fig. 2*C* and *D*), suggesting that materials coat the beads in a relatively uniform manner. Because H-2 molecules are about 50 Å in diameter, the antigen unavailable at the particle surface is probably located in bead crevices. Figure 2*E* shows for comparison by STEM a lymphocyte and H-2 antigen-bearing bead.

Pseudocytes and generation of specific effector CTL. The size of the beads and accessibility of incorporated H-2 antigen on the bead surface to papain suggested that they might provide an extremely effective substrate for the study CTL recognition. Recognition of H-2 alloantigens leads to proliferation of precursor CTL and their differentiation into effector CTL capable of lysing target cells that bear the same antigens. Previous work has shown that in vitro generation of the response is dependent on helper T cell production of lymphokines (5, 8), but

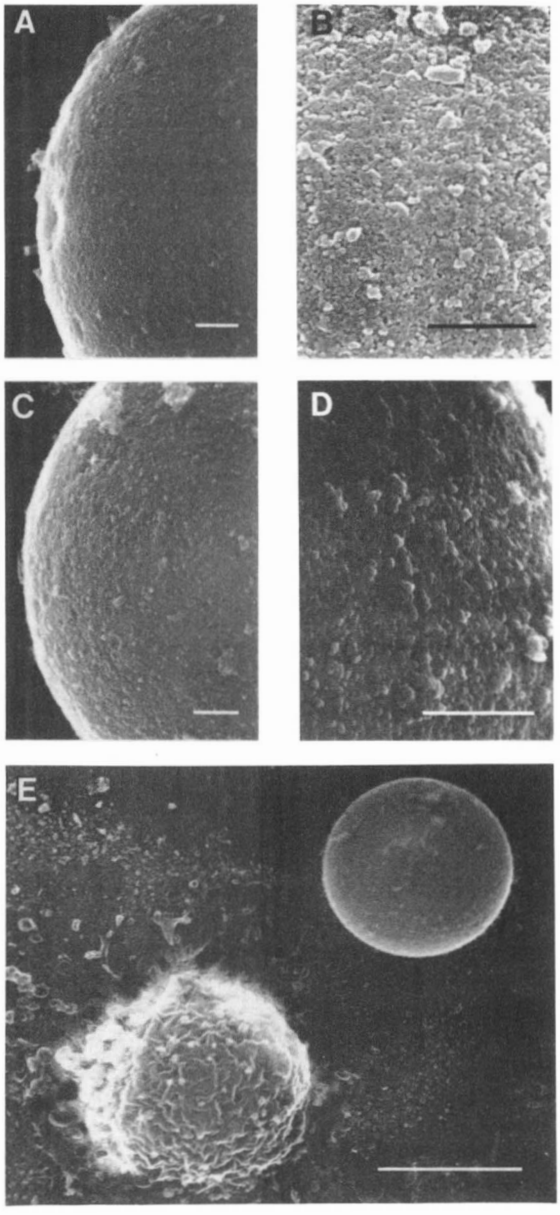

Figure 2. STEM of beads. The 5 μm beads were either untreated or coated by dialysis with lipid and H-2 antigen, and were allowed to settle on poly-L-lysine-treated coverslips for processing and visualization by STEM as described in *Materials and Methods*. Untreated beads, (*A*) 20,000× and (*B*) 50,000×; beads coated with 5 nmol cellular lipid and 6 μg H-2K^k antigen/10^7 beads, (*C*) 20,000× and (*D*) 50,000×; and (*E*) bead coated with 5 nmol cellular lipid and 12 μg H-2^d and a lymphocyte from an H-2^d anti-H-2^k secondary culture at 5000×. The size bar represents 0.5 μm (*A* to *D*) or 5 μm (*E*).

that this dependence can be eliminated by addition of lymphokine-containing supernatants from mitogen-stimulated rat lymphocytes. When an optimal amount of Con A supernatant is added, the magnitude of the lytic response depends only on the effectiveness of alloantigen recognition by precursor CTL (5). The response to H-2 antigen on beads, liposomes, and splenocytes was therefore assessed by measuring their ability to stimulate effector CTL generation in cultures containing optimal amounts of Con A supernatant.

Resting spleen cells from H-2^d and H-2^b mice previously immunized with RDM-4 (H-2^k) lymphoma cells were cultured for 5 days with different forms of alloantigen, and CTL lytic activity was assessed by using ^{51}Cr-labeled RDM-4 cells as targets in a 4-hr Cr-release assay (Fig. 3). The addition of H-2K^k-bearing beads or allogeneic splenocytes resulted in strong and comparable H-2^d anti-H-2^k responses, whereas no response was obtained in the absence of added alloantigen (Fig. 3*A*). No response occurred when beads coated with lipid but lacking H-2 antigen were added, indicating that stimulation by beads results from recognition of the H-2 antigen. The H-2K^k beads also stimulated an H-2^b anti-H-2^k response of comparable magnitude, and this response was also dependent on H-2 antigen expression on the bead surface (Fig. 3*B*).

Double-reciprocal experiments demonstrated that recognition of bead-borne class I antigen was immunologically specific, and provided additional evidence that beads do not have nonspecific mitogenic effects. Beads bearing H-2^k or H-2^d antigens were examined in d anti-k and k anti-d responses (Fig. 4). When splenocytes from AKR/J (H-2^k) mice, previously immunized with P815 (H-2^d) cells, were cultured for 5 days in the presence of beads bearing H-2^d antigen, effector CTL were generated that lysed P815 but not RDM-4 (H-2^k) target cells (Fig. 4*A* and *C*). Culture of the same cells with beads bearing self H-2K^k antigen resulted in no response when using either tumor cell target (Fig. 4*A* and *C*). Reciprocally, only H-2K^k beads stimulated a response by CD2F$_1$ (H-2^d) spleen cells previously immunized to RDM-4 (H-2K^k), and the resulting effectors were specific for target cells bearing the appropriate H-2K^k alloantigen (Fig. 4*B* and *D*). Spleen cells from C57BL/6 mice (H-2^b) previously primed to RDM-4 were also stimulated by only H-2^k-bearing beads, and not H-2^d- or H-2^b-bearing beads (data not shown).

The experiments above were performed by using beads made with an optimal amount of H-2 antigen (6 μg H-2K^k or 12 μg H-2^d or H-2^b per 10^7 beads) and an optimal number of beads per culture (10^6). Using twice as much H-2 antigen per bead did not increase responses, whereas a twofold decrease resulted in lower responses (data not shown). The response to antigen on beads was found to be relatively insensitive to the amount or composition of the lipid on the beads. H-2-bearing beads made by using synthetic lipids had comparable activity with those made by using lipids extracted from P815 tumor cells (Fig. 5),

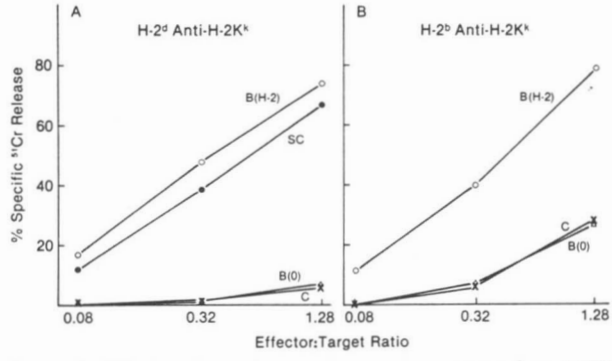

Figure 3. CTL stimulation by pseudocytes. Splenocytes from (*A*) CD$_2$F$_1$ (H-2^d) or (*B*) C57BL/6 (H-2^b) mice immunized 2 to 6 mo previously with RDM-4 (H-2^k) cells were cultured in the presence of Con A supernatant and alloantigen as described in *Materials and Methods*. After 5 days, effector CTL generation was assessed by measuring the release from ^{51}Cr-labeled RDM-4 cells. Antigens used were: 10^6 H-2K^k-bearing beads/culture (*B*(*H-2*), ○); 10^6 adherent cell-depleted A/J (H-2K^k/D^d) irradiated splenocytes/culture (*SC*, ●); 10^6 beads coated only with lipid (*B*(*O*), △), or no antigen (*C*, ×). Data are shown as percent specific ^{51}Cr release at varying effector to target ratios.

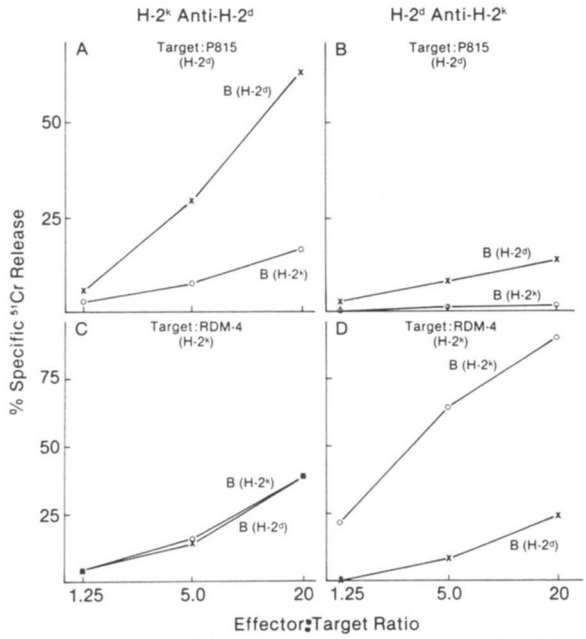

Figure 4. CTL stimulation by pseudocytes is specific. Splenocytes from AKR/J (H-2^k) mice immunized 2 to 6 mo previously with P815 cells (H-2^d), or from CD_2F_1 (H-2^d) mice immunized 2 to 6 mo previously with RDM-4 cells (H-2^k), were cultured in the presence of Con A supernatant and 10^6 beads per culture. Beads bore either H-2K^k (○) or H-2^d (×) antigen. After 5 days the cultures were assayed for lytic activity on both ^{51}Cr-labeled RDM-4 and P815 target cells. Data are expressed as percent specific ^{51}Cr release at varying effector to target ratios.

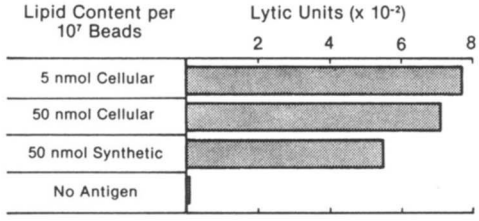

Figure 5. The effect of varying lipid composition on CTL stimulation by pseudocytes. Splenocytes from CD_2F_1 (H-2^d) mice immunized 2 to 6 mo previously with RDM-4 (H-2^k) cells were cultured with Con A supernatant and 10^6 beads. Beads were prepared from starting mixtures containing 6 μg H-2K^k/10^7 beads and either 5 nmol or 50 nmol cellular lipid/10^7 beads or 50 nmol synthetic lipid (DMPC:cholesterol, 98:2)/10^7 beads. H-2K^k incorporation was the same in each case. Cytotoxicity was assessed on day 5 by using ^{51}Cr-labeled RDM-4 target cells, and the results are expressed as lytic units per 10^6 cells.

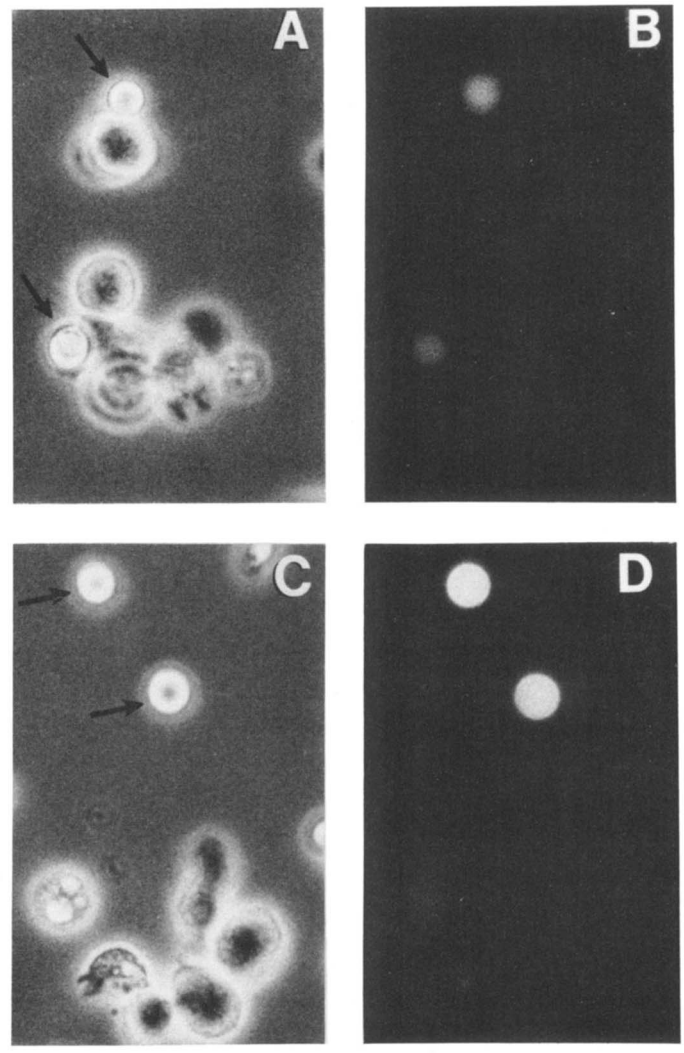

Figure 6. Phase-contrast and fluorescence photomicrographs of pseudocyte-cell conjugates. Splenocytes from CD_2F_1 (H-2^d) mice immunized 2 to 6 mo earlier with RDM-4 (H-2^k) cells were cultured for 5 days with H-2K^k-bearing beads and Con A supernatant. Cultures containing both cells and beads were split into aliquots and were incubated with either: (*A* and *B*) an irrelevant anti-NP idiotype mAb (R3-I) or (*C* and *D*) an anti-H-2K^k mAb (11-4.1), and then were stained with a fluoresceinated goat-anti-murine γ_{2a} antiserum as described in *Materials and Methods*. The samples were viewed at 400× under phase-contrast (*A* and *C*) or fluorescent (*B* and *D*) illumination. Beads (*arrows*) are 5 μm in diameter.

and varying the cell lipid to bead ratio from 5 nmol to 50 nmol per 10^7 beads had little or no effect on their stimulating activity (Fig. 5).

Pseudocyte-cell conjugate formation and reversal by specific antibodies. Beads and cells can be readily distinguished by light microscopy. Microscopic examination of immune spleen cell populations that had been cultured with beads bearing the immunizing alloantigen revealed clusters of proliferating cells on beads and bead-lymphocyte conjugates (Fig. 6*A*). Such conjugates are not observed when beads have no surface H-2 antigen or bear syngeneic or irrelevant third party antigen. The antigen dependence of the lymphocyte-bead association was demonstrated by mAb blockade.

Treatment of the H-2K^k-bearing beads with anti-H-2K^k mAb reversed conjugate formation (Fig. 6*C*), whereas treatment with an irrelevant mAb of the same subclass had no effect (Fig. 6*A*). Staining with a fluoresceinated anti-murine γ_{2a} mAb demonstrated the presence of H-2K^k on the beads, and the uniform appearance of the staining indicated that the antigen is not highly aggregated (Fig. 6*D*). In this experiment, there were 36 cells bound per 100 beads in the presence of the irrelevant antibody, and seven cells per 100 beads in the presence of anti-H-2K^k antibody (only cells in direct contact with beads were counted). The 36 bound cells represent about 10% of the cells present in this secondary in vitro CTL population, which is in the range of estimates for the number of effector CTL present in such populations (20). It should be noted that lymphocytes present in antigen-dependent conjugates with beads are likely to be effector CTL, but this has not been directly demonstrated. Experiments are in progress to examine the binding of cloned CTL lines to antigen-bearing beads.

Quantitative comparison of precursor CTL stimulation by alloantigen-bearing liposomes, pseudocytes, and cells. The response to H-2 antigen on liposomes was significantly less than to H-2 antigen on beads over a wide range of antigen concentrations (Fig. 7). To stimulate generation of responses of similar magnitude, 20 to

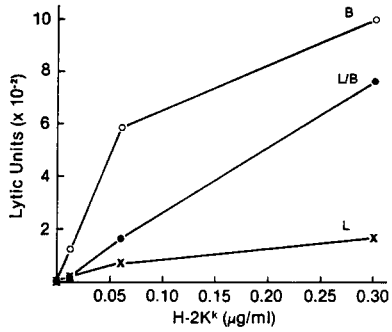

Figure 7. CTL stimulation by liposomes, liposome-coated beads, and pseudocytes bearing H-2K^k. Liposomes (*L*, ×) were prepared by mixing lipid and H-2K^k antigen in DOC, followed by dialysis to remove the detergent. Some of these liposomes were incubated with untreated 5 μm beads; the beads were pelleted and washed to yield liposome-coated beads (*L/B*, ●). Pseudocytes (*B*, ○) were formed by mixing H-2K^k (6 μg H-2K^k/10^7 beads), lipid and beads in DOC/TBS and dialyzing. Details of the preparations are given in *Materials and Methods*. Splenocytes from CD_2F_1 (H-2^d) mice immunized 2 to 6 mo previously with RDM-4 (H-2^k) cells were cultured with Con A supernatant and varying amounts of the different antigens (increasing numbers of beads or liposomes; expressed as final H-2K^k concentration in culture) and were assayed for lytic activity on day 5 by using ^{51}Cr-labeled RDM-4 cells. Data are expressed as lytic units per 10^6 cells.

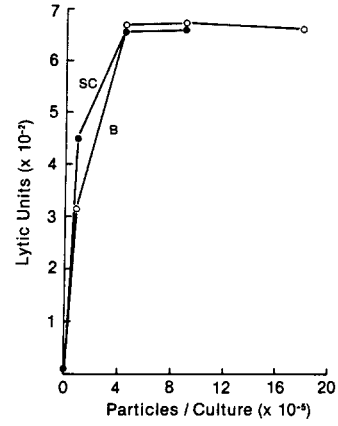

Figure 8. Comparison of CTL stimulation by allogeneic spleen cells and pseudocytes. Splenocytes from CD_2F_1 (H-2^d) mice immunized 2 to 6 mo previously with RDM-4 cells (H-2^k) were cultured with Con A supernatant and increasing numbers of H-2K^k-bearing beads (*B*, ○) or irradiated A/J (H-2K^k/D^d) splenocytes depleted of adherent cells (*SC*, ●). Cytotoxicity was assessed by using ^{51}Cr-labeled RDM-4 target cells after 5 days, and data are shown as lytic units/10^6 cells.

50 times more H-2 antigen must be added to culture when liposomes rather than beads are used. This is probably due to the dramatic difference in size, and thus potential area for interaction, between liposomes (<0.1 μm diameter) and beads (5 μm diameter), which provide a surface area that is at least 2500 times larger. Support for the suggestion that size is the critical difference between liposomes and pseudocytes was provided by experiments examining beads coated with preformed liposomes. Small, unilamellar liposomes bearing H-2K^k were formed by dialysis and were then incubated with untreated beads in TBS at a ratio of 7 μg H-2 (in liposomes) to 6 × 10^6 beads. Approximately 70% of the H-2 liposomes bound to the beads, all of which remained bound after three washes in serum-containing medium. Liposomes coated onto beads were dramatically more effective than free liposomes in stimulating a response (Fig. 7), but not as effective as comparable amounts of H-2 antigen incorporated onto beads by dialysis. Thus, while coating cell size beads with liposomes provides an alternative means of enhancing antigen recognition, the dialysis procedure remained the method of choice.

It is more difficult to compare quantitatively the stimulation by beads and allogeneic spleen cells on the basis of H-2 antigen dose due to uncertainties in estimating the amount of H-2 antigen on cells and to the fact that cells may continue to express new surface antigen after addition to culture. Beads and cells can be compared, however, on the basis of particle number added to the cultures. When this was done, a close correspondence was found between the number of beads and number of irradiated, adherent cell-depleted allogeneic cells needed to stimulate generation of a given level of response (Fig. 8). Despite some variation in the absolute magnitude of the cytolytic response between animals, beads were consistently found to be as efficient as equal numbers of irradiated cells in numerous experiments (data not shown). Thus, alloantigen on cell-sized beads bearing only lipid and class I antigen appears to be as effectively recognized by precursor CTL as alloantigen on intact cells.

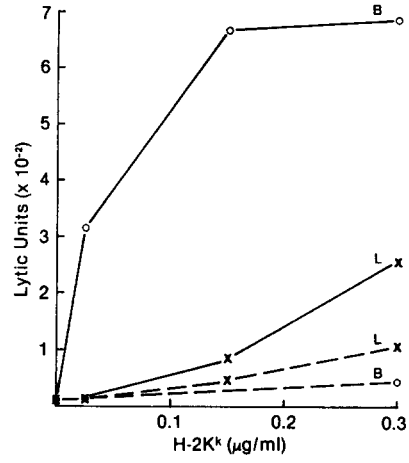

Figure 9. CTL stimulation by pseudocytes is completely dependent upon the addition of lymphokines. Splenocytes from CD_2F_1 (H-2^d) mice immunized 2 to 6 mo previously with RDM-4 cells (H-2^k) were cultured with H-2K^k-bearing beads (*B*, ○) or liposomes (*L*, ×) in the presence (——) or absence (- - - -) of added Con A supernatant. Beads were made by using 6 μg H-2K^k and 5 nmol lipid per 10^7 beads, and liposomes by using 1 μg H-2K^k per 20 nmol lipid. Antigen dose was varied by varying the number of beads or liposomes added, and is expressed as the final concentration of antigen in the culture. Cytotoxicity was assayed by using ^{51}Cr-labeled RDM-4 cells after 5 days, and data are reported as lytic units/10^6 cells.

CTL generation by pseudocytes is completely dependent upon added lymphokines. The magnitude of the responses shown above provided a direct measure of antigen recognition by precursor CTL, because an optimal amount of Con A supernatant was added in each case (5). In the absence of added factors, the generation of effector CTL is limited by accessory cell presentation of alloantigen to helper T cells and subsequent endogenous factor production (5, 8). H-2 antigen-bearing liposomes can be taken up by Ia-positive accessory cells and the alloantigen presented to helper T cells (8). As a result, a CTL response to liposomes can be seen in the absence of added factors (Fig. 9). In contrast, the response to H-2 antigen-bearing beads is much more highly dependent upon the addition of factors, even when the beads are added at twice the dose needed to generate maximal responses in the presence of added factors (Fig. 9). Thus, recognition of H-2 antigen on beads by precursor CTL is

dramatically more effective than on liposomes, and generates a much stronger response if factors are added, but stimulates less well than liposomes in the absence of added factors. That the 5 μm beads are unable to support a response in the absence of added factors indicates that their H-2 antigen is stable during culture. This is consistent with the observation that H-2 antigen is readily detectable by immunofluorescence on the bead surface after 5 days in culture with cells at 37°C (Fig. 6).

Pseudocytes do not stimulate primary CTL responses. Although H-2 antigen-bearing liposomes or isolated PMV are able to stimulate secondary in vitro allogeneic responses, they are poor stimulators of responses by spleen cells from nonimmune animals (17). There are several possible explanations for the inability of subcellular antigen to stimulate strong primary responses.

Precursor CTL from nonimmune animals might bind antigen with lower avidity than primed precursor CTL, and thus may have more stringent requirements for multivalency and surface area of interaction. However, although the cell-sized, antigen-bearing beads were dramatically better than membranes or liposomes in stimulating secondary responses, they were unable to generate a primary response. No primary response to H-2K^k-bearing beads could be detected either with or without added Con A supernatant, whereas the same beads and supernatant preparations stimulated a secondary response comparable with that seen with intact allogeneic spleen cells (Table II). Even the weak response seen to PMV was not seen when using the beads (Table II).

Alternatively, nonimmune precursor CTL might need to interact with other surface proteins in addition to class I antigen. The results of preliminary experiments when using beads bearing both purified class I antigen and plasma membrane proteins from A20 (bearing allogeneic class II molecules) or P815 cells have not supported this suggestion. No primary response was obtained in either case (data not shown).

Generation of a primary response might require soluble factors not present in Con A supernatant and not generated in response to beads. Support for this suggestion was provided in two ways. Nonimmune CD_2F_1 (H-2^d) responder spleen cells were placed in culture with either H-2K^k-bearing beads or irradiated C57BL/6 (H-2^b) splenocytes as third party stimulators, or a mixture of both stimuli, and effector CTL generation was assayed 5 days later. Cells from cultures stimulated with just H-2K^k-bearing beads did not lyse H-2^k-bearing target cells, whereas those from cultures stimulated with third party allogeneic (H-2^b) cells gave only marginal (presumably cross-reactive) lysis (Table II). However, significant lysis was obtained from cultures stimulated with both allogeneic (H-2^b) cells and H-2K^k-bearing beads (Table II). Thus, some response to antigen on beads occurred in the presence of an ongoing response to third-party alloantigen on stimulator cells.

Another type of mixing experiment showed that alloantigen-bearing PMV from RDM-4 (H-2^k) cells were able to stimulate marginal responses when added to culture alone, but significant responses were generated when they were added to culture along with H-2K^k antigen-bearing beads (Table II). Thus, both cases in which alloantigen-bearing beads are present along with co-stimulators that can activate the helper T cell pathway show that a primary response can result from recognition of antigen on the beads. This suggests that activation of naive precursor CTL may require soluble factors that are not required for a secondary response and are not present in the Con A supernatants we have used.

TABLE II

Generation of CTL in nonimmune and immune spleen cultures[a]

Antigen[b]	Response (lytic units per 10^6 cells)			
	Nonimmune		Immune	
	+Con A Sn	−Con A Sn	+Con A Sn	−Con A Sn
None	<5	<5	<10	<10
H-2K^k beads	<5	<5	670	48
A/J (H-2K^k) SC	65	70	675	500
C57BL/6 (H-2^b) SC	6	<5	—[c]	—
H-2K^k beads + C57BL/6 (H-2^b) SC	20	<5	—	—
RDM-4 (H-2^k) PMV	7	<5	—	—
H-2K^k beads + RDM-4 (H-2^k) PMV	16	<5	—	—

[a] Responder cells were splenocytes from CD_2F_1 (H-2^d) mice either not primed (*Nonimmune*) or primed 2 to 6 mo previously with RDM-4 (H-2^k) cells (*Immune*). Cells were placed in culture with the indicated antigen with or without the addition of Con A supernatant (*Con A Sn*), and lytic activity was assayed 5 days later by ^{51}Cr released from RDM-4 target cells.

[b] Antigens: spleen cells (SC) from A/J (H-2^a) and C57BL/6 (H-2^b) were irradiated and used at 10^6 cells per culture. H-2K^k-bearing beads were used at 10^6 beads per culture. RDM-4 PMV were used at 2.5 μg/ml. When mixtures of antigens were added to cultures these same doses were used.

[c] This analysis was not performed.

DISCUSSION

T cells recognize antigen on the surfaces of other cells, and the tens of thousands of receptor and antigen molecules on the juxtaposed cell surfaces provide the potential for very highly multivalent interaction. The notion that T cell recognition involves interaction of multiple low affinity receptors (21) and requires highly multivalent antigen for avid binding and for triggering of responses is supported by cases in which binding of nominal antigen to T cells has been demonstrated (22, 23). Previous work examining stimulation of in vitro CTL responses by subcellular antigen indicated that the physical size of the antigen-bearing structure, and thus the potential multivalency of interaction, is a critical parameter in determining the level of response obtained (6). Small unilamellar liposomes bearing affinity-purified class I antigens have provided an effective means of defining some of the requirements for antigen recognition and triggering of precursor CTL with respect to the structure of antigens (4) and the role of lymphokines in the generation of a response (5). At the same time, the use of liposomes has a number of limitations. They are readily taken up by accessory cells (5, 8) and have the potential to fuse into the surface membranes of cells in culture. Thus, although the available evidence strongly suggests that precursor CTL recognize antigen on the liposome surface (6), this has not been directly demonstrated. Furthermore, it appeared likely that the small size of liposomes limited effective antigen recognition by restricting the multivalency of interaction.

These considerations prompted our efforts to prepare cell-sized artificial membranes bearing class I antigens. As described in this report, this has been accomplished by using 5 μm diameter, silica beads modified with C_{18} alkyl chains, which are sold commercially as packing for reverse phase high pressure liquid chromatography col-

umns. Mixing of lipid, H-2 antigen, and beads in DOC-containing buffer and dialyzing to remove the detergent results in all of the lipid and protein becoming associated with the beads (Table I and Fig. 1), and approximately 40% of the H-2 antigen is exposed at the bead surface (Table I). The amount of lipid and H-2 incorporated onto the beads could be readily controlled by varying the ratios of the components in the starting mixture, and the extent of H-2 incorporation and surface exposure were relatively insensitive to the amount of lipid used (Fig. 1). The feasibility of protein and lipid incorporation by dialysis was suggested by the work of Ishiguro and Nakanishi (24), which demonstrated that glycophorin and lipid could be incorporated onto alkylated glass slides by dialysis.

The H-2-bearing, cell-sized beads, termed pseudocytes, were able to stimulate the generation of secondary CTL responses with the same specificity as alloantigen-bearing spleen cells (Figs. 3 and 4), provided that lymphokines (Con A supernatant) were added. Quantitative comparisons showed H-2 antigen on beads to stimulate much more effectively than on liposomes (Fig. 7). In fact, pseudocytes were as effective as alloantigen-bearing spleen cell stimulators when compared on the basis of the response obtained as a function of the number of particles (cells or beads) added to culture (Fig. 8). Enhanced recognition of antigen on beads, in comparison to liposomes, appears to be due to the larger size of the antigen-bearing structure, because coating beads with preformed H-2-bearing liposomes resulted in a considerable increase in stimulating activity (Fig. 7).

The generation of a response to H-2 antigen on beads was found to be completely dependent upon the addition of lymphokines to the cultures (Fig. 9). Thus, unlike liposomes (5, 8), the beads cannot be taken up by accessory cells to a sufficient extent to allow processing and presentation of alloantigen to helper T cells, nor is sufficient antigen shed from the beads to allow helper T cell activation even when two times more bead-bound antigen is added than is needed for maximal precursor CTL triggering (Fig. 9). These results strongly indicate that precursor CTL directly recognize the class I antigen on the surface of the bead. Additional evidence for direct recognition was provided by visual examination of cultures stimulated with antigen-bearing beads. Lymphocyte-bead conjugates were seen in cultures containing effector CTL, provided that the beads bore the appropriate alloantigen, and these conjugates were eliminated if antibody specific for the H-2 antigen on the beads was added (Fig. 6). Thus, the cell-sized beads make it possible to study the molecular parameters important in precursor CTL recognition without the possibly confounding effects of uptake and presentation of the antigen by other cells in the cultures. Furthermore, the ease of identifying and quantitating lymphocyte-bead conjugates by microscopy should make it possible to use this approach to study antigen binding by cloned CTL lines.

The beads used in these studies have 50 to 110 Å pores (Fig. 2). The bead surface has a smoother appearance and the pores are less apparent after binding of lipid, suggesting that the lipid may form a relatively continuous surface coat on the bead. The fraction of bead-bound H-2 antigen that is inaccessible to papain is probably located within the pores. The nature of the surface lipid coat has not been determined and may vary depending upon the amount of lipid put on the beads. At low levels of lipid, a bilayer may form, the lower half of it consisting of the covalently linked C_{18} alkyl chains on the bead. At high levels of lipid, it is likely that multiple lipid lamellae may be present. If so, these variations in structure do not have any dramatic effect upon accessibility of H-2 antigen on the bead surface to papain (Table I) or bead recognition by precursor CTL (Fig. 5). The susceptibility of the surface H-2 antigen to papain cleavage, its binding by anti-H-2 antibody, and its recognition by precursor CTL indicate that the antigen maintains its native conformation and is associated with the lipid-coated surface of the bead in a manner similar to its association with the cell surface. Indeed, the diffuse surface staining of the beads with fluoresceinated reagents specific for the H-2 antigen indicate that antigen is arrayed in a relatively uniform manner over the surface of the beads, and is not highly aggregated (Fig. 6). Work is in progress to additionally characterize the bead surface structure, and to examine the effects of changing the bead size and the alkyl chain length on antigen incorporation and precursor CTL recognition.

In addition to size, mobility of the class I antigen on the bead surface may influence its recognition. In general, class I antigens on cell surfaces are mobile and diffuse in the plane of the membrane. In contrast, class I antigens do not diffuse when incorporated into supported planar membranes prepared by incubating liposomes with either alkylated glass slides or rigorously cleaned slides (9). Photoleaching measurements showed the H-2 protein in these membranes to be immobile, whereas lipid probes incorporated into the same membranes were freely mobile (9). In contrast, wheat germ agglutinin-agarose binding to glycophorin incorporated onto alkylated glass slides by dialysis indicated that the glycophorin was mobile in the membrane (24). Whether class I antigen is mobile on the surface of the bead-supported membranes described in this report has not yet been determined.

Binding of effector CTL to planar membranes bearing class I antigen has been reported (25), as has stimulation of in vitro CTL generation by such membranes (9). Class II antigen-bearing planar membranes and membranes on 100 μm glass beads, along with nominal antigen, have been shown to trigger lymphokine production by helper T cell hybridomas (10, 11), as have class II alloantigens on 100 μm glass beads (26). Although these membranes provide some advantages for studying antigen recognition by T cells, they are also limited with respect to characterization of the membranes, the ability to manipulate the antigen, and the large amounts of antigen needed for preparation. Indeed, 100 μm glass beads are 8000 times larger (mm^3) than a 5 μm diameter cell, and thus are poorly amenable to microscopic or FACS analyses.

The antigen-bearing beads described in this report overcome many of these limitations. They can be easily made and quantitated by using a hemocytometer. Their lipid and protein composition can be measured by including radiolabeled tracers, and their surface composition can be determined by fluorescence microscopy (Fig. 6) and quantitated by FACS analysis (manuscript in preparation). The beads can essentially be handled like cells, with the advantages of having well-defined, readily var-

ied, and easily quantitated composition. Furthermore, the very small amount of antigen required to prepare large numbers of beads (<6 μg per 10^7 beads) should make this approach useful for studying recognition of surface proteins that cannot be readily isolated in as large amounts as native class I and II antigens. For example, class I or II antigens altered by exon shuffling or site-directed mutagenesis, and expressed in cells, might be purified by affinity chromatography and their recognition studied in the absence of confounding effects such as the levels of expression on the transfected cell or the contributions of host cell proteins.

The inability of pseudocytes to stimulate the generation of a primary allogeneic CTL response (Table II) was somewhat surprising in light of their effectiveness in stimulating secondary responses. The lack of response was not due to inhibitory effects of the beads, nor does it appear to result from requirements for the presence of additional surface proteins on the antigen-bearing membranes. The results obtained thus far indicate that the generation of a primary response may involve soluble factors not required for the generation of a secondary response. This is suggested by several observations. When H-2K^k-bearing beads are present in cultures that also contain third party allogeneic spleen cells, a response to the H-2K^k is seen, whereas no response is found in the absence of third party stimulators (Table II). Production of nonantigen-specific soluble factors in response to intact stimulator cells, but not to pseudocytes, could explain these results. Alloantigen-bearing plasma membranes are able to stimulate a weak primary response (Table II) (17), whereas pseudocytes cannot. When H-2K^k-bearing beads are present in cultures that also contain alloantigen-bearing PMV, a significant response is seen (Table II). Because antigen on small membrane vesicles and liposomes can induce lymphokine production (Fig. 9) (5, 8), and because this does not occur when antigen-bearing beads are used (Fig. 9), factor generation could also explain these findings. Although supernatants from Con A-stimulated rat lymphocyte cultures contain all of the soluble factors necessary to support a secondary response to pseudocytes (Fig. 9), the addition of Con A supernatants to primary cultures does not result in a response to pseudocytes (Table II). Thus, if the inability of pseudocytes to stimulate a primary response is due to the absence of a required factor(s), then it is likely to be a factor that is not required in a secondary response and is not present in the Con A supernatants we have used.

Several groups have reported evidence showing that in vitro generation of effector CTL requires soluble factors in addition to interleukin 2 (27–30), but these factors have not been well characterized. The complete lack of response to pseudocytes in the absence of soluble factors, even when unfractionated responder spleen cells are used, should make these antigen-bearing beads a very effective means of pursuing study of these factors and their roles in CTL activation.

Pseudocytes bearing H-2K^k have been shown in this report to trigger secondary allogeneic responses as effectively as spleen cell stimulators. It follows that only class I alloantigenic determinants are absolutely required for activation of the majority of splenic precursor CTL. These results also demonstrate that induction of the cytolytic response does not require nonantigen-specific interaction of the T cell receptor or accessory molecules with molecules other than H-2 antigen.

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